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SPAWNING, DEVELOPMENT, AND ACQUISITION OF ZOOXANTHELLAE BY *TRIDACNA SQUAMOSA* (MOLLUSCA, BIVALVIA)

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Abstract

Spawning and development of the clam *T. squamosa* was investigated in Belau, Western Caroline Islands in February and March 1980. Five of the six species of tridacnid clams spawned in response to various stimuli, but only *T. squamosa* released mature eggs. Factors influencing gonad development and spawning are discussed.

Development of *T. squamosa* was followed from post-fertilization to post-metamorphosis, with emphasis on the acquisition of the zooxanthella *Symbiodinium (=Gymnodinium) microadriaticum* Freudenthal. The symbiotic algae were not seen in either the fertilized eggs or trochophore stages. We found that all strains of *S. microadriaticum* introduced to veliger clams were taken into the stomach via the mouth. Veligers ingested motile zooxanthellae more readily than non-motile ones. Within 2–9 days *after* metamorphosis, zooxanthellae moved by an unknown mechanism into the developing siphonal tissues. Most of these zooxanthellae appeared to be in spaces, probably the developing haemal sinuses. However, in some cases it was difficult to tell if the zooxanthellae were intra- or extracellular. Survival and growth of veligers and juveniles with zooxanthellae was greater than those without zooxanthellae. Juveniles with zooxanthellae can survive and grow in Millipore-filtered seawater with light as the sole energy source for over 10 months, illustrating the phototrophic aspect of the association.

Our observations may have practical application pertinent to spawning, development, and growth of tridacnid clams in the context of commercial mariculture.

Introduction

There has been much interest in the biology of tridacnid clams, particularly in regard to their symbiosis with algae, since Yonge's (1936) classic study. The tridacnids and the heart shell *Corculum cardissa*, members of the Superfamily Cardiacea, are the only marine bivalves known to harbor endosymbiotic dinoflagellates (Brock 1888, Boschma 1924, Yonge 1936, Kawaguti 1950, 1968). These algae have been identified as *Symbiodinium (=Gymnodinium) microadriaticum* (Freudenthal, 1962) (hereafter also referred to as zooxanthellae), allegedly the same species of dinoflagellate that inhabits all reef-building corals and many anemones, gorgonians, hydroids, and jellyfishes (*cf.* Freudenthal 1962, Taylor 1969, 1974; Loeblich and Sherley, 1979; Schoenberg and Trench, 1980a, b, c). The zooxanthellae in tridacnids are most numerous in the hypertrophied tissues of the clams' siphon, but may also be found in the heart, stomach, digestive gland, and intestine (Mansour 1946a, b, c; Kawaguti 1968; Goreau *et al.*, 1973; Trench *et al.*, 1981).

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Yonge (1936) hypothesized that the algae were probably passed from parents to offspring. However, subsequent studies by LaBarbera (1975) and Jameson (1976) have shown that neither eggs nor sperm released from tridacnids contain zooxanthellae, implying that each generation must acquire their complement of symbionts from the environment.

The latter observations raise a number of intriguing questions. First, when are the zooxanthellae acquired during the development of the clam? Larval tridacnids maintained in unfiltered seawater and observed just after metamorphosis already had zooxanthellae in their siphon tissues (LaBarbera, 1975; Jameson, 1976). Whether these algae were acquired before or after metamorphosis, or are needed for metamorphosis, was not determined. Dependence of an organism on a symbiont for completion of its life cycle may be more common than originally thought (see Provosoli *et al.*, 1968; Taylor, 1971b; Edson, 1981). Other examples are *S. microadriaticum* and the rhizostome jellyfish *Mastigias papua* (Sugiura, 1963, 1964) and *Cassiopeia xamachana* (Trench *et al.*, 1981; Trench, 1981).

Second, what is the mechanism of acquisition of the algae? In the scyphistomae of *C. xamachana,* zooxanthellae are taken in via the mouth and are phagocytozed by endoderm cells (Trench, 1980; Trench *et al.*, 1981). The zooxanthellae take up permanent residence inside the digestive cells of the coelenterate and somehow avoid digestion by the host (Trench, 1979). The situation in the Tridacnidae is somewhat different. Zooxanthellae in the siphon tissues are not found inside cells. Instead they lie free in the blood sinuses. Morton (1978) suggested that infection might be by "accidental invasion" of the clam, the zooxanthellae somehow moving through the "external surface of the mantle". The other possible route is through the digestive system. It was originally thought that this was unlikely because of the efficient digestive capabilities of herbivorous filter-feeding bivalves (Yonge, 1936; 1975). However, several studies have shown that some algae can pass through the digestive system of bivalves without being digested (Coe, 1948; Dean, 1958; Haven and Morales-Alamo, 1966; Hildreth, 1980). In the Tridacnidae, apparently healthy zooxanthellae (Mansour, 1946b, c), including motile forms (Ricard and Salvat, 1977) capable of fixing $^{14}CO_2$ and being cultured (Trench *et al.*, 1981) are commonly extruded in fecal pellets.

Do tridacnid clams establish a symbiosis only with certain species or strains of zooxanthellae? Three species of dinoflagellates have been described as endosymbionts in marine invertebrates; two have been placed in the genus *Amphidinium* and the remaining one is referred to as *S. microadriaticum* (Freudenthal, 1962; Taylor, 1971a, 1973, 1974). A vast majority of the symbiotic coelenterates contain the latter symbiont. Distinct strains of *S. microadriaticum* have recently been distinguished by biochemical and morphological criteria (Schoenberg and Trench, 1980a, b). Selectivity in the uptake of strains of *S. microadriaticum* has been experimentally documented (Schoenberg and Trench, 1980c; Trench 1981; Trench *et al.*, 1981). Tridacnid clams are found in symbiotic associations *only* with *S. microadriaticum* (Taylor, 1969). This observation, coupled with the fact that naturally occurring aposymbiotic adult clams have never been reported, suggests selectivity in the process of acquisition leading to specificity in the association. Discrimination during the uptake of different strains of *S. microadriaticum* by the Tridacnidae has not been previously addressed.

In this study we investigated these three questions concerning the acquisition of *S. microadriaticum* by larval and juvenile *T. squamosa.* We found that all strains of *S. microadriaticum* introduced to veliger clams were taken in via the mouth. They made their way by an unknown mechanism into the siphonal tissues

after metamorphosis. Motile zooxanthellae were more apt to be taken into veliger stomachs and may lead to higher veliger survival than non-motile zooxanthellae. Survival and growth of veligers and juveniles was greater with zooxanthellae than without them.

Materials and Methods

Experimental organisms

Adult tridacnid clams, except for *Tridacna gigas,* were collected from their natural habitat on the reefs of Belau, Western Caroline Islands, and transported in containers of sea water to the Micronesian Mariculture Demonstration Center (MMDC) on the Island of Malakal. Specimens of *T. gigas* were collected in 1978–1979 by personnel at the MMDC and were living on the reef adjacent to the laboratory. All individuals of the other five species were maintained in outdoor cement tanks in unfiltered running seawater until spawning experiments began.

Release of gametes

Three methods of initiating spawning were attempted: introduction of macerated gonads from a clam of the same species, placing clams in warm water, and introduction of hydrogen peroxide. In addition, spontaneous (non-induced) spawnings were documented. Water flow in the tanks was stopped during spawning experiments. All attempts on *T. gigas* were performed *in situ.*

We attempted to induce spawning with macerated gonads the day after collection and, in some cases, after periods ranging from 1 week to 1 month after collection. In practice, portions of gonad were dissected from one individual, macerated, and scattered in tanks of water containing other members of the same species. For induction of spawning with warm water we set clams in 30 l fiberglass tubs and let the water heat up to 30–35°C in the sun for periods ranging from 1–8 h. Small clams were placed in containers with 12 l of seawater and spawning was induced with 7.5×10^{-2} *M* hydrogen peroxide brought to pH 9.1 with the addition of 2*M* TRIS (see Morse *et al.*, 1977). For larger clams, 6% hydrogen peroxide was squirted directly into the incurrent siphon. The actual concentration reaching the clam was not determined.

Fertilization

Freshly spawned eggs were obtained by rinsing a clam spawning eggs in seawater and placing it in a tub of 20 l of seawater uncontaminated by sperm. The clam was allowed to go through 1–3 spawning reactions (see Wada, 1954) before being moved back to the cement holding tank. Sperm-laden water (~50 mls), derived from other clams, to minimize the chance of self fertilization, was added to the container of eggs and mixed thoroughly (see LaBarbera, 1975).

Maintenance of larvae

Fertilized eggs were pipetted into sterile Petri dishes containing 0.22 μm Millipore-filtered seawater (MFSW). All subsequent developmental stages were maintained in MFSW in the laboratory, at ambient air temperatures (24–31°C) and illumination. Seawater was changed approximately every 4 days. Except for zooxanthellae, there were no other additives to the cultures.

TABLE I

Host sources of isolates of zooxanthellae taken into the gut of veligers of Tridacna squamosa. *Strain designations correspond to those of Schoenberg and Trench (1980a). "U," uncharacterized.*

Cultured	(Class, Phylum)	Strain
Cassiopeia xamachana	(Scyphozoa, Coelenterata)	C
*Zoanthus sociatus**	(Anthozoa, Coelenterata)	Z
*Zoanthus solanderi***	(Anthozoa, Coelenterata)	U
Aiptasia tagetes	(Anthozoa, Coelenterata)	A
Aiptasia pallida	(Anthozoa, Coelenterata)	U
Tridacna gigas	(Bivalvia, Mollusca)	T
Tridacna maxima	(Bivalvia, Mollusca)	U
Freshly Isolated		
Tridacna squamosa	(Bivalvia, Mollusca)	U
Tridacna gigas	(Bivalvia, Mollusca)	U
Tridacna maxima	(Bivalvia, Mollusca)	U
Tridacna derasa	(Bivalvia, Mollusca)	U
Tridacna crocea	(Bivalvia, Mollusca)	U
Hippopus hippopus	(Bivalvia, Mollusca)	U
Aiptasia sp.	(Anthozoa, Coelenterata)	U
Mastigias papua	(Scyphozoa, Coelenterata)	U

* Strain "Z," isolated in Jamaica in 1974 by D. A. Schoenberg.
** Strain "U," isolated in Jamaica in 1979 by S. S. Chang.

Introduction of zooxanthellae

Previously cultured and freshly isolated zooxanthellae were introduced to cultures of veligers 2 days after spawning (see Table I). Strains of cultured *S. microadriacticum* were originally isolated from various invertebrate hosts, including sea anemones, jellyfishes, and two species of *Tridacna*. They were grown in the artificial culture medium ASP-8A (McLaughlin and Zahl, 1957, 1959, 1966; Ahles, 1967) for at least 1 year before use in experiments. Approximately 0.2 ml wet-packed cells were added to Petri dishes of veligers. Freshly isolated zooxanthellae were from all six tridacnid clams, a local anemone, *Aiptasia* sp., and the jellyfish *Mastigias papua*. Freshly isolated zooxanthellae were obtained by scraping a piece of clam siphon or jellyfish manubrium tissue with a scalpel in a Petri dish of MFSW until a brown suspension made up of released zooxanthellae and small pieces of animal tissue covered the bottom. This suspension was allowed to stand for 5 min before the supernatant, containing animal tissue, mucus, and some zooxanthellae, was poured off. Zooxanthellae adherent to the bottom of the Petri dish were rinsed gently with MFSW, squirted off the bottom, centrifuged, and combined until approximately 0.2 ml of wet-packed cells were accumulated. Algal pellets released from *Aiptasia* sp. were collected, washed twice with MFSW, and centrifuged to a 0.2 ml wet-packed pellet before use in infection experiments.

Microscopy

A light microscope with a calibrated occular micrometer was used to measure the length (longest dimension) of the larval and juvenile *Tridacna* and to determine presence or absence of zooxanthellae. Some sorting and veliger counts were made using a dissecting microscope.

Clams were fixed for electron microscopy in Karnovsky's (1965) fixative for 1

h, rinsed twice with 0.2 *M* sodium cacodylate buffer (pH 7.3), and post-fixed with 1% osmium in 0.2 *M* sodium cacodylate buffer for 1 h. Specimens were stored in 70% ETOH at 4°C prior to preparation for transmission electron microscopy (TEM). Specimens were dehydrated in ETOH and transferred through 5 changes of propylene oxide before being embedded with Araldite 6005. Sections were made on a LKB Ultramicrotome V with glass knives, stained with uranyl acetate and lead citrate, and observed and photographed on a Siemens Elmiskop I.

Results

Spawning

All three artificial methods induced spawning, but none produced viable eggs (Table II). However, two instances of spontaneous spawning of *T. squamosa* produced viable eggs. These occurred on consecutive days (2–3 March 1980) in the morning, coincident with a new moon and a falling tide, in a group of clams collected 3 weeks earlier. Spawning began at 1000 h and continued for over 9 h. Sperm were produced first, for up to 6 h with much individual variation. This was followed by the release of eggs 3–9 h after the beginning of sperm release. All individuals (n = 55) in the tank spawned sperm. However, the number of clams releasing eggs was not determined.

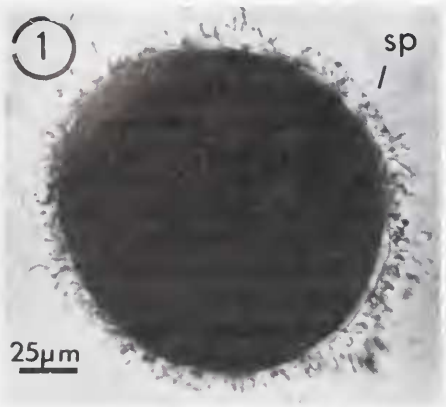
The addition of macerated gonads to water containing adult clams almost always induced spawning with release of sperm within 1–5 min. Release of sperm continued 0.5–6 h. Many of the clams thus tested showed weak spawning behavior, producing low concentrations of sperm over a 2 h period. *T. derasa* spawned in response to the addition of hydrogen peroxide. Within 15 min these clams reacted to the addition of 6% hydrogen peroxide into the incurrent siphon with vigorous discharge of sperm and the production of large amounts of mucus, continuing 4–6 h. Dissections showed large gonads with many sperm but no mature eggs.

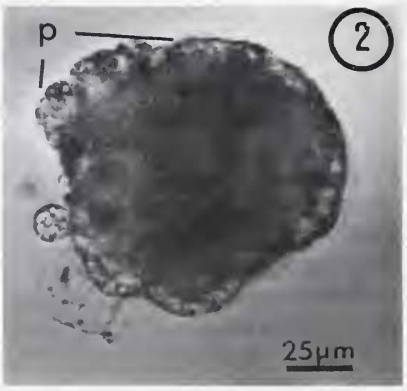
Table II

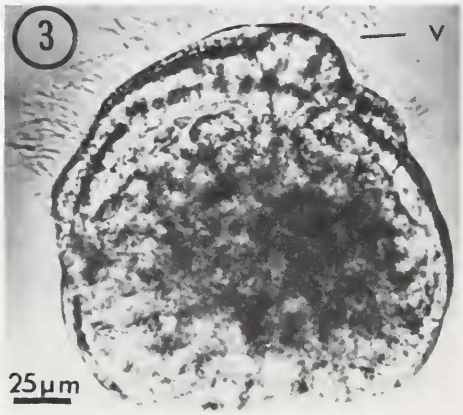
Number of spawning events for four methods of induction during the period February 19 to March 17, 1980. s = sperm spawned, e = eggs spawned, n = number of attempts. W = Winter, Sp = Spring, Su = Summer, F = Fall.

Species	Reported breeding season	Total number tested	Method											
			Macerated gonads			Elevated temperature			Peroxide			Spontaneous		
			s	e	n	s	e	n	s	e	n	s	e	n
Tridacna squamosa	W[1]	55	3	0	4	0	0	1	0	0	1	3	2	3
Tridacna maxima	W[2]	50	4	0	4	1	0	2	0	0	1	1	0	1
Tridacna derasa	Sp[3]	15	1	0	1	—	—	—	1	0	2	—	—	—
Tridacna crocea	Su[4]	40	0	0	1	0	0	2	0	0	2	1	0	1
Tridacna gigas	F[5]	6	0	0	1	—	—	—	0	0	1	—	—	—
Hippopus hippopus	Su[6]	14	2	0	2	—	—	—	—	—	—	1	0	1

[1] Wada (1954), Rosewater (1965), Hardy and Hardy (1969), LaBarbera (1975), Beckvar (1981).
[2] LaBarbera (1975), Jameson (1976).
[3] Jameson (1976), Beckvar (1981).
[4] Jameson (1976).
[5] Beckvar (1981).
[6] Stephenson (1934), Jameson (1976), Yamaguchi (1977), Beckvar (1981).







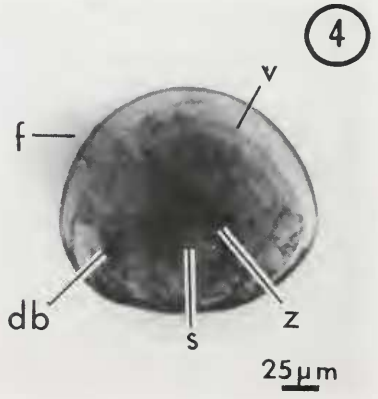


FIGURE 1. Egg spawned from *T. squamosa*. Note sperm (sp) around the outside of the egg.

FIGURE 2. Trochophore larva of *T. squamosa*. A band of cilia, known as the prototroch (p), is visible at the anterior end of the animal.

FIGURE 3. Day 3 veliger of *T. squamosa*. v = velum.

FIGURE 4. Pediveliger of *T. squamosa*. *The foot* (*f*) *and velum* (*v*) are both present at this stage of development. Zooxanthellae (z) can be seen lining the stomach. Usually 3–4 dark bodies (db) are present in the posterior portion of the animal.

Induction of spawning by raising the water temperature was generally unsuccessful. Clams tended to close and release mucus as water temperature rose, indicating unsatisfactory conditions.

Larval development

Fertilized eggs (Fig. 1) passed through the 8–16 cell stage in 3–6 h. Ciliated gastrulae appeared 6–9 h post-fertilization. Trochophore larvae (Fig. 2) were first observed between 12–20 h after fertilization. Veligers (Fig. 3) appeared 24–30 h after fertilization. Two days after fertilization veligers had a well developed velum, a stomach and an esophagus. The intestine at this point is still a solid cylinder

(LaBarbera, 1975), developing a lumen on day 3. Pediveligers (Fig. 4) appeared on day 10, and alternately swam and crawled on the bottom for at least a day before metamorphosis. There was extreme variation in times of pediveliger development; some were observed up to 29 days after fertilization (see Table V). No zooxanthellae were observed in any of these developmental stages in animals maintained in MFSW.

Infection with algal symbionts

Cultured and freshly isolated *S. microadriaticum* (see Table I) were introduced into Petri dishes of veligers on day 2. Zooxanthellae were not seen in the alimentary tract of the veligers until the end of day 3. All strains of zooxanthellae tested were seen in the stomachs of the clams during the veliger stages, except those derived from pellets extruded by the local *Aiptasia* sp. (Table III). The different strains of zooxanthellae exhibited different degrees of maximum motility (cf. Fitt *et al.*, 1981). A comparison of motility levels and presence of zooxanthellae taken into the stomach (Table III) suggested that these two phenomena might be related.

To test whether motile zooxanthellae are more likely than non-motile zooxanthellae to be ingested by veligers, the following experiment was performed. Twenty uninfected veligers were placed in petri dishes of zooxanthellae from *T. squamosa* during the day when motile forms were present. After 7 h, 90% of the veligers had zooxanthellae in their stomachs. Only 10% of veligers had zooxanthellae in their stomachs when the same experiment was done at night, when no motile zooxanthellae were present. To establish if these differences were due to nocturnal changes in feeding behavior of the veligers, the experiment was repeated with cultured zooxanthellae isolated from *A. pallida*. This alga is motile in the morning and non-motile in the afternoon (Fitt *et al.*, 1981). Results corroborated the first experiment. Between 0600 h and 1300 h, 90% of the 20 uninfected veligers ingested zooxanthellae; while between 1400 h and 2100 h, only 10% of the veligers were found with zooxanthellae in their stomachs.

To determine if different strains of zooxanthellae affect growth of larval clams, we measured shell length over time. Measurements of day 8 veligers and pediveliger

TABLE III

Relative motility of zooxanthellae and the frequency of observations (% days) of zooxanthellae in the gut of veligers of Tridacna squamosa. *Relative maximum motility in seawater is designated by: + = less than 1% motile cells, ++ = 1–25%, +++ = 25–50%, ++++ = >50%. Observations on gut contents were begun on day 4 and ended when metamorphosis began on day 11. See text for further details.*

Host source of cultured zooxanthellae	Motility	% Days	Host source of freshly isolated zooxanthellae	Motility	% Days
Cassiopeia xamachana	+++	100	*Tridacna squamosa*	++++	100
*Zoanthus sociatus**	+	88	*Tridacna gigas*	+++	100
*Zoanthus solanderi***	++	100	*Tridacna derasa*	++	100
Aiptasia tagetes	+	25	*Tridacna crocea*	++	100
Aiptasia pallida	+++	100	*Tridacna maxima*	++	100
Tridacna gigas	++	100	*Hippopus hippopus*	++++	100
Tridacna maxima	++	100	*Aiptasia* sp.***	+	0

* Strain "Z." ** Strain "U." *** <10 motile cells seen during all observations.

TABLE IV

Length (μm) of veligers (day 8) and pediveligers of Tridacna squamosa *maintained in Millipore-filtered seawater with various isolates of zooxanthellae. SD = standard deviation.*

Host sources of zooxanthellae	Day 8 veligers			Pediveligers			
	Mean	SD	n	Mean	SD	n	Age (days)
Cultured							
Cassiopeia xamachana	158	6	10	169	13	31	11–29
*Zoanthus sociatus**	158	9	10	166	7	15	10–18
*Zoanthus solanderi***	153	9	10	—	—	—	—
Aiptasia tagetes	160	—	1	167	9	19	11–20
Aiptasia pallida	158	6	10	169	9	45	11–26
Tridacna gigas	162	6	10	161	10	19	11–17
Tridacna maxima	156	4	10	160	9	19	11–19
Freshly Isolated							
Tridacna squamosa	158	7	10	162	9	19	10–20
Tridacna gigas	—	—	—	165	5	13	11–17
Tridacna maxima	—	—	—	162	9	19	11–17
Tridacna derasa	—	—	—	167	6	19	10–17
Tridacna crocea	—	—	—	171	8	14	10–20
Hippopus hippopus	—	—	—	169	8	15	11–15
Aiptasia sp.	157	5	10	—	—	—	—
Mastigias papua	—	—	—	168	4	2	11–12
Control (no zooxanthellae)	152	6	10	159	7	14	11–20
Range	152–162			159–169			

* Strain "Z." ** Strain "U."

stages showed no significant ($p > 0.05$) differences in length between animals exposed to different strains of *S. microadriaticum* (Table IV). Veligers and pediveliger larvae (without zooxanthellae) were typically smaller than animals with zooxanthellae, although the differences were not significant.

Different strains of zooxanthellae were added to Petri dishes of 600 uninfected veligers on day 3. Survival was monitored on days 7, 8, 9, and 10 by determining the proportion of live and dead animals from a subsample of 30 animals. Veligers exposed to zooxanthellae freshly isolated from *T. squamosa* had significantly ($p < 0.05$, comparison of slopes) higher survival rates than veligers exposed to cultured zooxanthellae from *A. pallida, C. xamachana, Zoanthus solanderi, T. gigas,* and *T. maxima* (Fig. 5). Animals with the above strains of zooxanthellae all had significantly ($p < 0.05$, comparison of means) higher survival after 7 days than veligers with zooxanthellae isolated from *Z. sociatus* (strain Z), *A. tagetes* and the controls (without zooxanthellae). The experiment was terminated on day 10, when pediveligers began metamorphosing to juvenile clams.

Metamorphosis in the Tridacnidae is a gradual process, involving less swimming and more crawling as the shell gets heavier and the velum degenerates. The appearance of a statocyst at the base of the foot (Fig. 12) indicates the completion of metamorphosis (Jameson, 1976). The first juveniles were seen on day 11 (Table V). Minimum time to metamorphosis of veligers exposed to different strains of

zooxanthellae was typically 11–15 days. However, there was wide variation in the times of development (see Table IV). Metamorphosis occurred in some clams as late as day 30. Clams without zooxanthellae metamorphosed at the same times as those with zooxanthellae. Mortality was high for all groups of clams, regardless of strain of zooxanthellae. Less than 5% of the veligers alive on day 3 survived through to complete metamorphosis, regardless of the experimental conditions.

No zooxanthellae were seen in the mantle region of newly metamorphosed clams (Fig. 6), in spite of the fact that zooxanthellae were common in the stomachs of

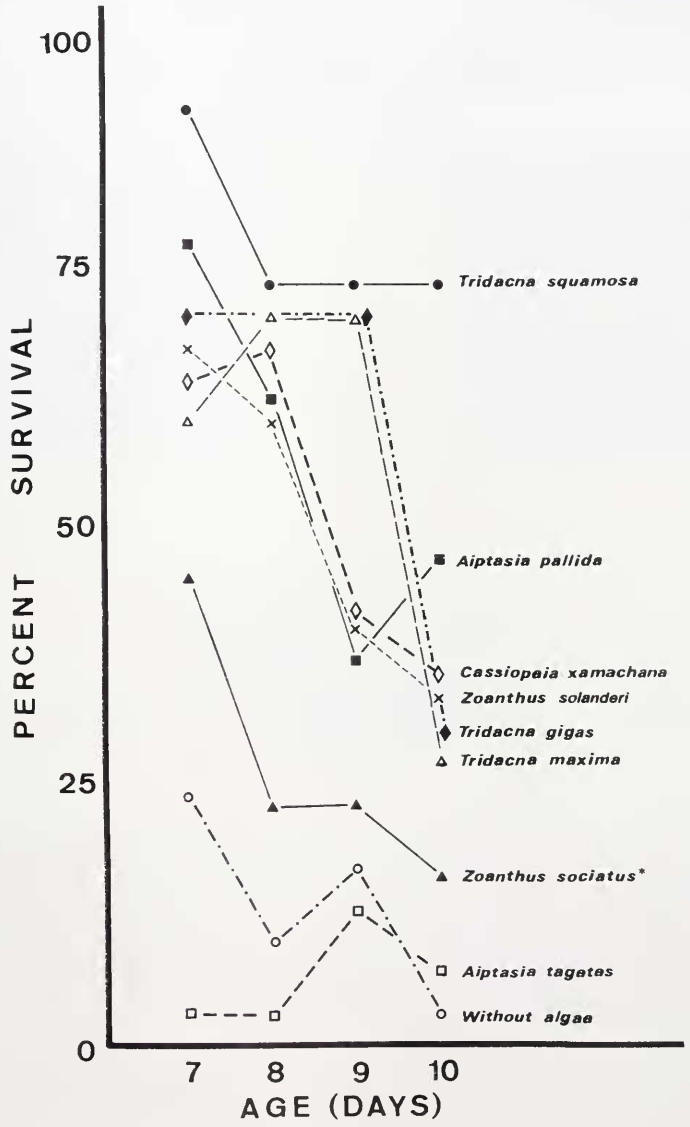


FIGURE 5. Percent survival of veligers of *T. squamosa* over 7 days in Petri dishes containing zooxanthellae isolated from the various hosts indicated on the right of each line. The experiment was terminated on day 11, when metamorphosis began. * = strain "Z", ** = strain "U".

TABLE V

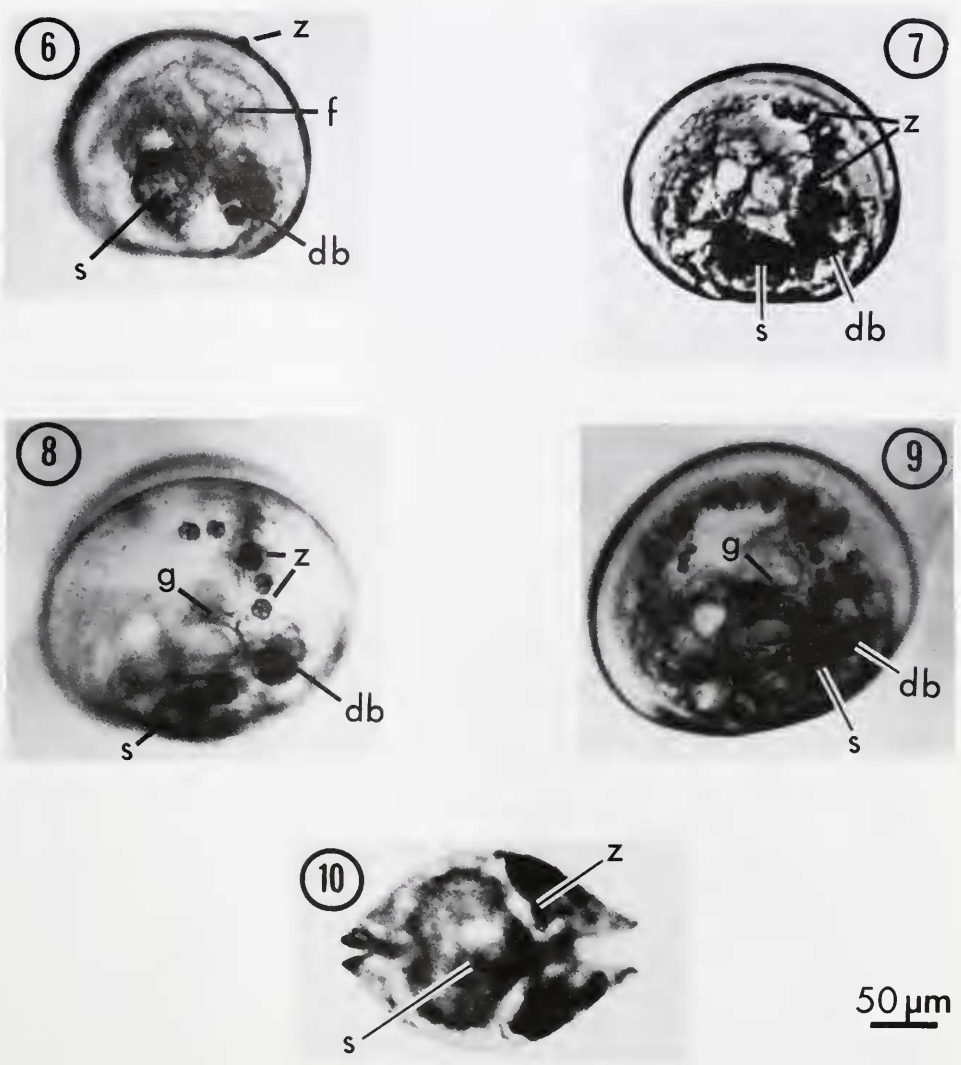
Developmental timetable of late veliger and juvenile Tridacna squamosa *maintained in Petri dishes of cultured and freshly isolated zooxanthellae from various hosts. Numbers indicate minimum age in days.*

Host source of zooxanthellae	Pediveliger	Juvenile	Zooxanthellae in siphon tissues	Days between first juvenile sighting to first sighting of zooxanthellae in siphon tissue
Cultured				
Cassiopeia xamachana	11	14	18	4
*Zoanthus sociatus**	10	11	18	7
*Zoanthus solanderi***	11	—	—	—
Aiptasia tagetes	11	20	20	0***
Aiptasia pallida	11	15	18	3
Tridacna gigas	11	15	17	2
Tridacna maxima	11	15	18	3
Freshly Isolated				
Tridacna squamosa	10	13	17	4
Tridacna gigas	11	15	17	2
Tridacna maxima	11	11	20	9
Tridacna derasa	10	14	17	3
Tridacna crocea	10	14	16	2
Hippopus hippopus	11	14	19	5
Control (no zooxanthellae)	11	17		

* Strain "Z."
** Strain "U."
*** Only 1 juvenile survived metamorphosis; it was first found with zooxanthellae half-way across well-developed siphon tissues, indicating that metamorphosis took place several days previous to this observation.

these clams. Between 2–7 days after metamorphosis (Table V) zooxanthellae appeared in the ventral mantle regions of the animal as two rows of cells, one on either side of the clam (Figs. 7, 11). This line of algae usually passed between or just anterior to three to four dark bodies located just posterior to the digestive gland and stomach/style sac (Figs. 7, 8). These zooxanthellae appeared healthy and many were seen dividing (Fig. 8). It took 1–3 weeks for the zooxanthellae to spread throughout the siphon tissue (Figs. 9, 12). This phenomenon was the same, regardless of the strain of zooxanthellae present.

Electron microscopy did not reveal any clear differences in morphology or location of different strains of zooxanthellae in juvenile clams. Zooxanthellae in the stomach did not appear to be inside cells (Fig. 11); some appeared healthy while others showed signs of degeneration. It is not known whether unhealthy zooxanthellae were being digested or undergoing autolysis (*cf.* Fankboner, 1971; Muscatine, 1973; Trench *et al.*, 1981). Zooxanthellae were observed outside the



FIGURES 6–9. Hinge (dorsal) is down, anterior is left. Bar = 50 µm. FIGURE 6. Newly metamorphosed juvenile of *T. squamosa*. Zooxanthellae (strain, "T", isolated from *T. gigas*) can be seen inside the clam as dark spheres in the stomach (s) and outside of the clam next to the shell. There are no zooanthellae in the ventral mantle regions of the clam. Other symbols as in Fig. 4. Figure 7. A row of zooxanthellae (strain "U", isolated from *Z. solanderi*) in a 19-day-old *T. squamosa* juvenile, extending from the posterior stomach (s) region to the ventral mantle portion of the clam. Other symbols as in Fig. 4. Figure 8. Dividing zooxanthellae (strain "T", isolated from *T. gigas*) in the mantle tissues bordering the gills (g) of a 19-day-old *T. squamosa*. Other symbols as in Fig. 4. Figure 9. Zooxanthellae (strain "C", isolated from *C. xamachana*), extending across the entire ventral margin of a 21-day-old *T. squamosa*. Other symbols as in Figs. 4, 8. Figure 10. A dorsal view of a 19-day-old *T. squamosa* juvenile showing the relation between the heart-shaped stomach (s) lined with dark spherical zooxanthellae, and the rows of zooxanthellae on both sides of the posterior portion of the clam. Bar = 50 µm.

stomach either immediately adjacent to the stomach (Fig. 13), and the base of the foot (Fig. 12), or in the mantle tissues bordering the shell (Figs. 11, 14). These algae were probably in developing digestive diverticula and haemal sinuses. Most

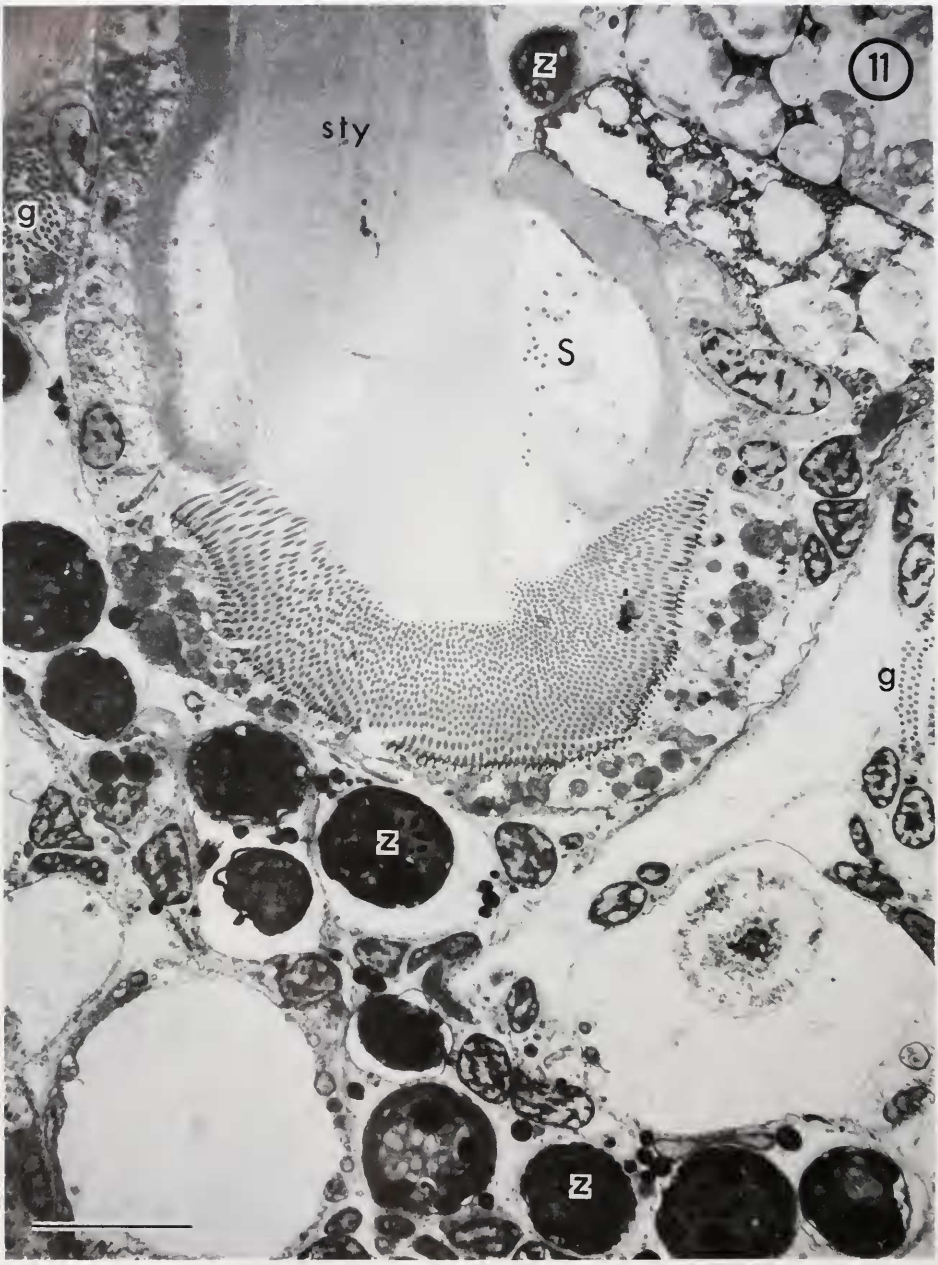
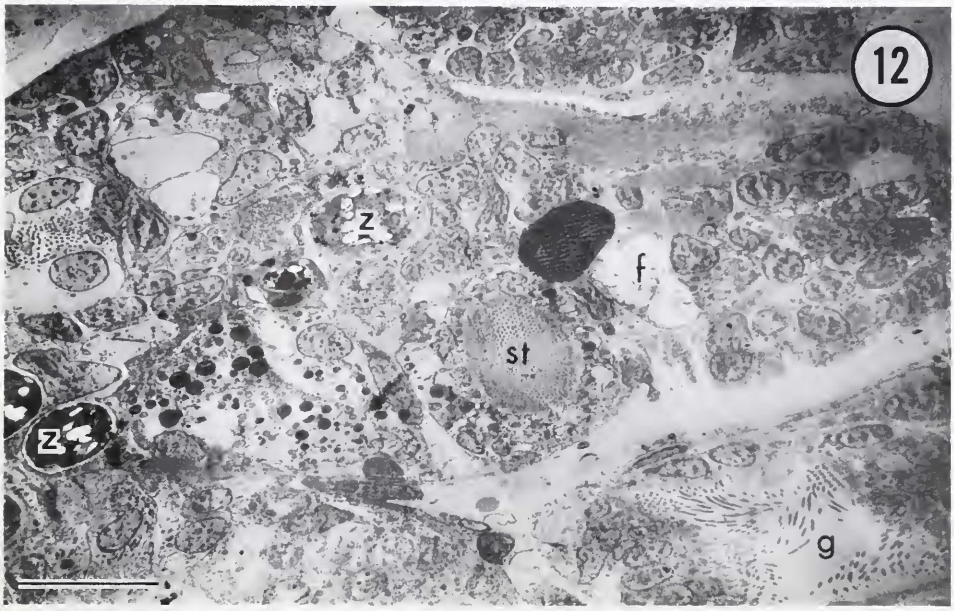


FIGURE 11. Oblique longitudinal section of a 20-day-old *T. squamosa* juvenile, showing the stomach (s) containing the style (sty) and a zooxanthella (z). Two rows of zooxanthellae extend from an area just posterior to the stomach to the left and right mantle regions of the clam just posterior to the gills (g). All zooxanthellae are strain "U", isolated from *A. pallida*. Bar = 10 μm.



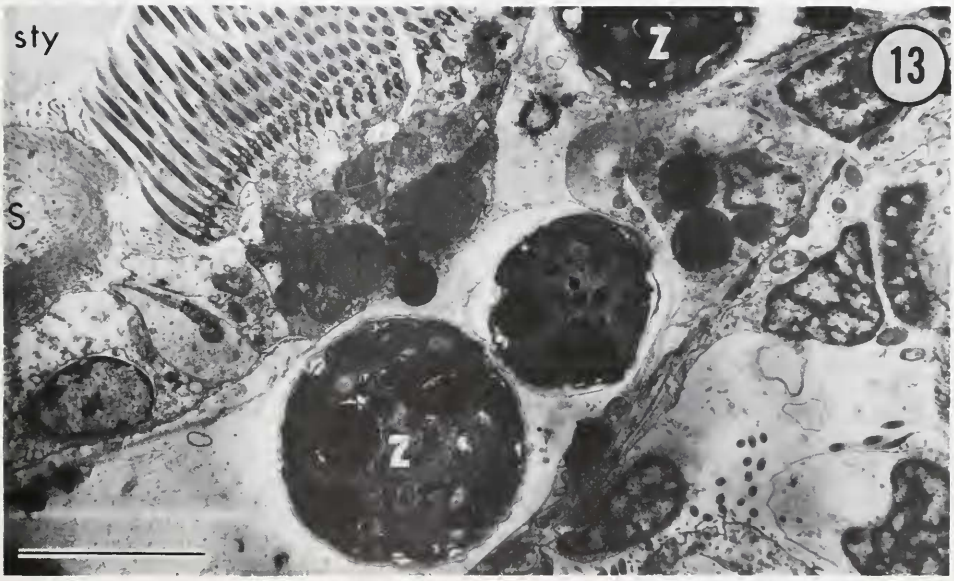


FIGURE 12. Transverse section of a 16-day-old *T. squamosa* juvenile with zooxanthellae (z) (strain "U", isolated from *T. crocea*) in the dorsal stomach region at the left and at the base of the foot (f) near the statocyst (st). A portion of the gills (g) is visible on one side of the clam. Bar = 10 μm.

FIGURE 13. Zooxanthellae (strain "U", isolated from *A. pallida*) in an open space (probably developing digestive diverticulae or haemal sinuses) just posterior to the stomach (s) in a 20-day old juvenile *T. squamosa*. Other symbols as in Fig. 11. Bar = 5 μm.

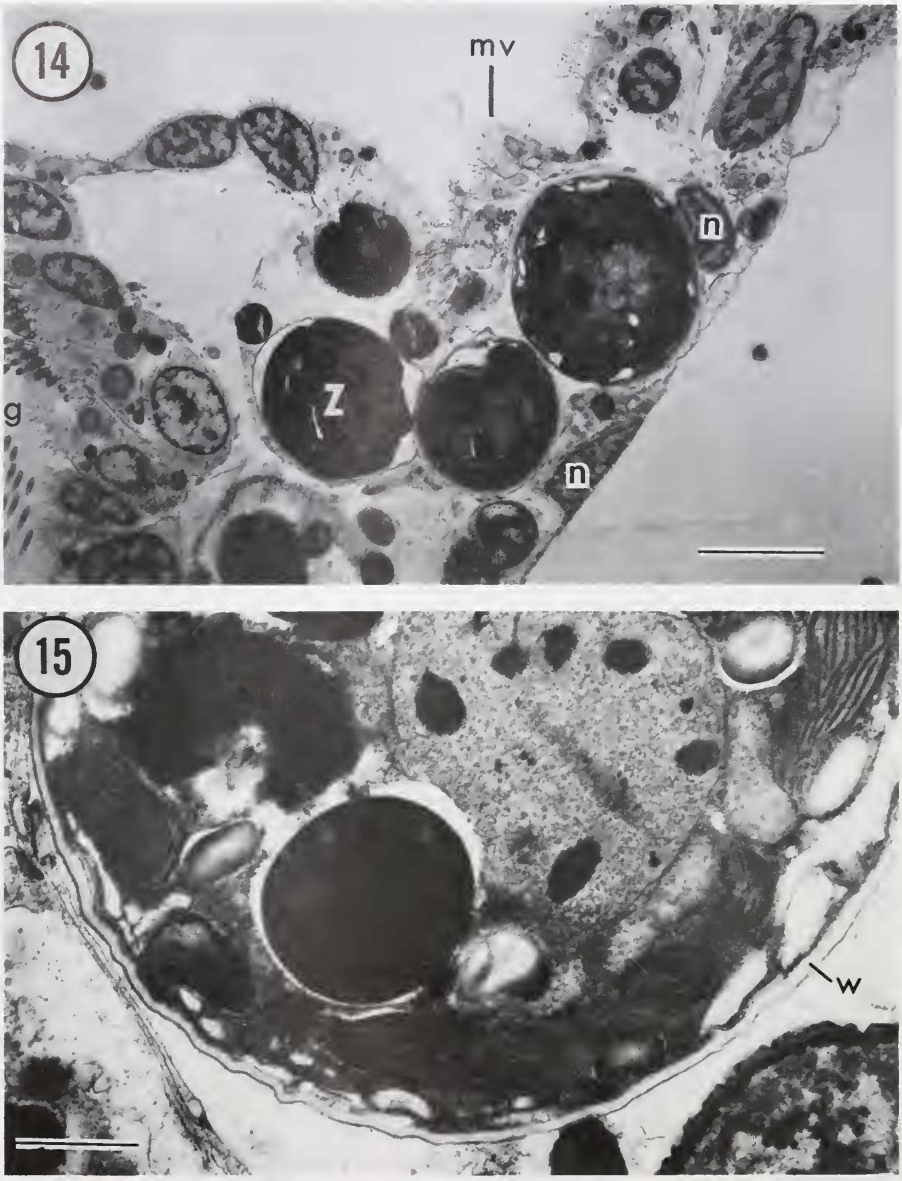
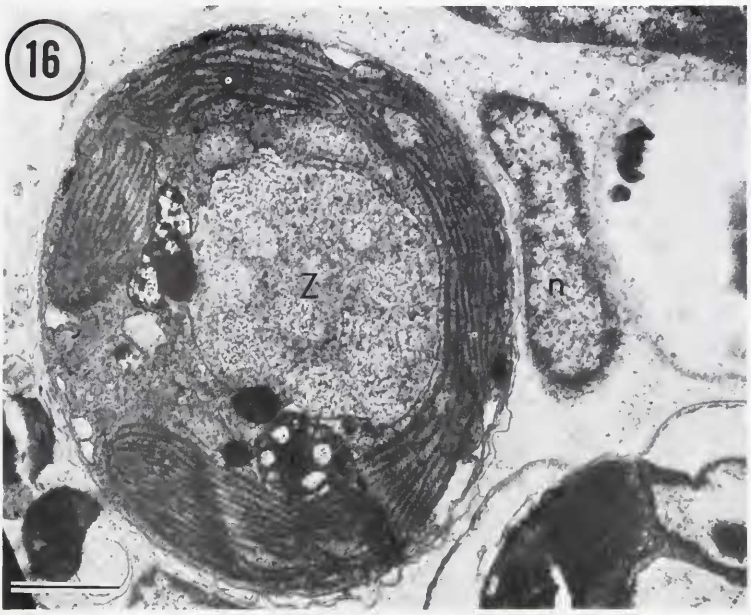


FIGURE 14. Zooxanthellae (z) (strain "U", isolated from *A. pallida*) in the mantle region of a 20-day-old juvenile *T. squamosa*. Note border of microvilli (mv) and close approximity of host cells and host cell nuclei (n). Bar = 5 μm.

FIGURE 15. A zooxanthella (strain "U", isolated from *A pallida*), lying free, probably in developing haemal sinuses in the mantle region of a 20-day old juvenile *T. squamosa*. w = zooxanthellar cell wall. Bar = 1 μm.

of these zooxanthellae appear to lie free in a space or tubule lined with host cells (Figs. 13, 14, 15). In some cases, however, it is difficult to tell from electron micrographs if the zooxanthellae are extra- or intracellular (Figs. 16, 17). Some of the zooxanthellae outside the stomach also appeared degenerate.



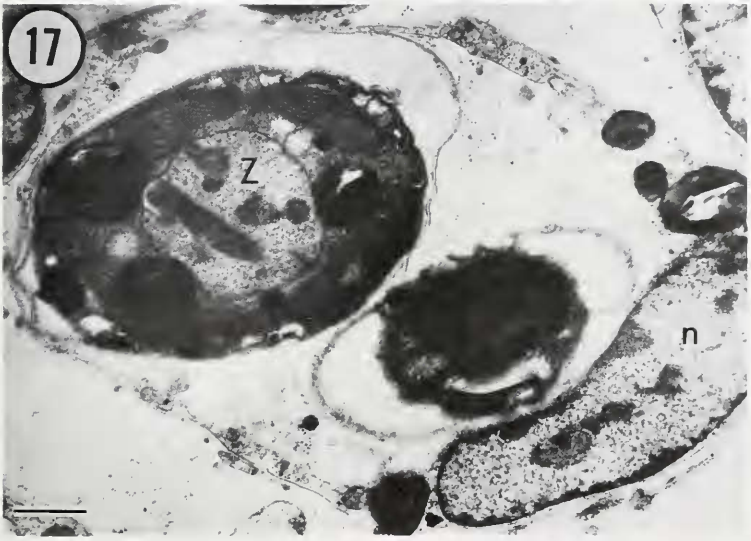


FIGURE 16. A zooxanthella (z) (strain "U", isolated from *A. pallida*) in the mantle region of a 20-day-old juvenile *T. squamosa* showing surrounding host tissue and a host nucleus (n). Bar = 1 μm.

FIGURE 17. A zooxanthella (z) (strain "U", isolated from *A. pallida*) apparently surrounded by a host cell in the mantle region of a 20-day-old juvenile *T. squamosa*. Other symbols as in Fig. 16. Bar = 1 μm.

Juveniles without zooxanthellae did not live beyond 3 weeks. Juveniles with zooxanthellae lived substantially longer; about 15 clams increased in length between 4–9 μ/day when maintained in MFSW in our laboratory over a 10 month period with light (60 $\mu E \cdot m^{-2} \cdot sec^{-1}$) as the sole known energy source.

Newly metamorphosed clams with zooxanthellae had growth rates comparable

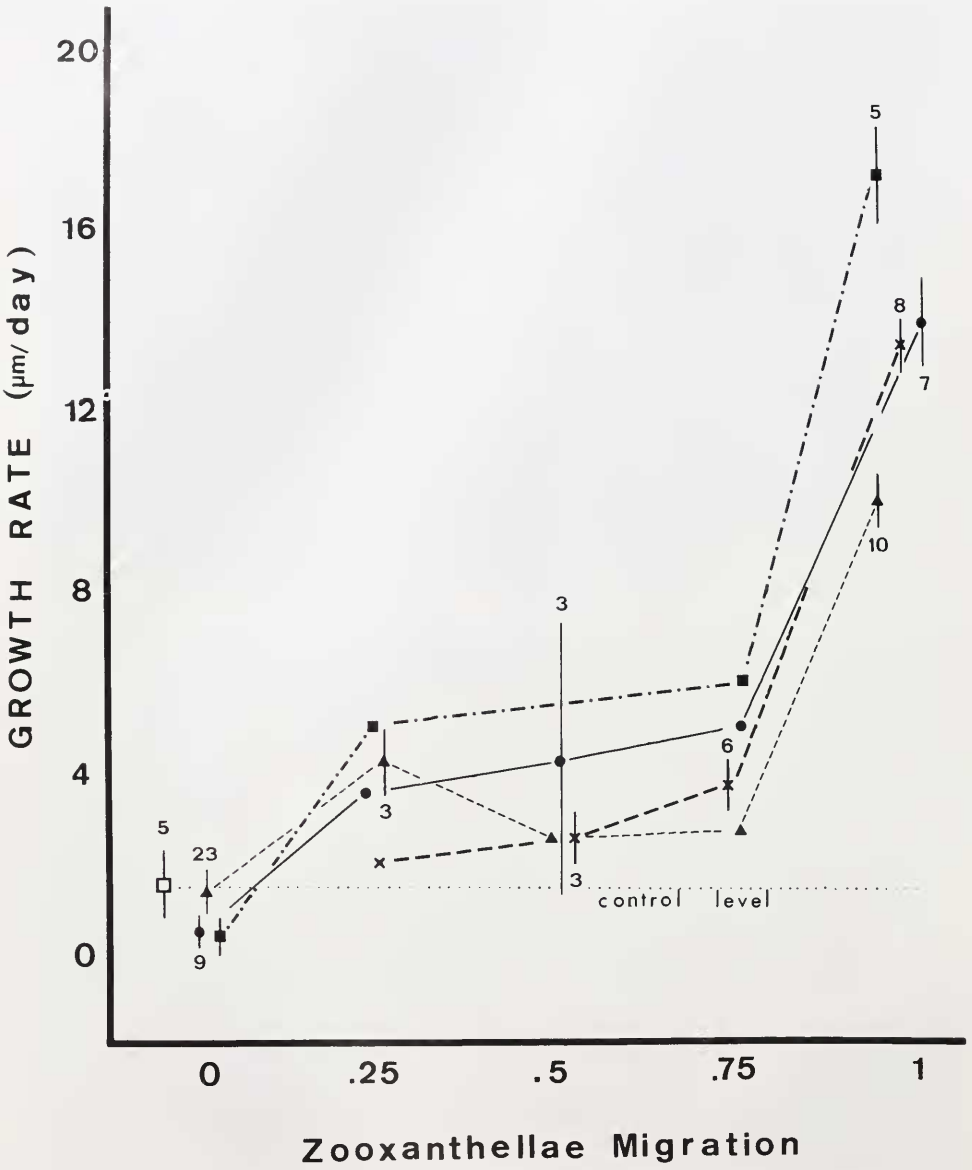


FIGURE 18. Growth rates of juvenile *T. squamosa* with different strains of zooxanthellae extending across their mantle regions. Scale for zooxanthellae migration: 0 = no zooxanthellae in mantle region (see Fig. 6), 0.5 = zooxanthellae extend about half way across ventral region of clam (see Fig. 7), 1 = zooxanthellae extend entirely across ventral portion of the clam (see Fig. 9). Clams contained zooxanthellae isolated from *H. hippopus* (■). *T. squamosa* (●), *A. pallida* (×), and *T. crocea* (▲). Control clams without zooxanthellae (□). Bars represent ±1 standard error, with "n" noted on one side of the bar.

to control clams without zooxanthellae. As the zooxanthellae moved across the central portion of the clam with the developing siphon tissues, the growth rates of the clams with zooxanthellae increased (Fig. 18). When zooxanthellae had moved

completely across the siphon tissues, growth rates were significantly ($p < 0.05$) higher than controls, ranging from 6-20μm per day, and were comparable to those obtained by Jameson (1976). At this point, juveniles of *T. squamosa* infected with different isolates of zooxanthellae demonstrated different growth rates.

Discussion

Spawning

The term "spawning" in bivalves usually refers to the release of sperm and/or eggs. Since tridacnids are hermaphroditic (Wada, 1952), spawning can refer to either the release of sperm or eggs, or both, from the same clam. Unfortunately, published accounts of tridacnid spawning often do not distinguish between these alternatives. In our experiments, we observed spawning in five of the six species of tridacnid clams. However, only 2 of the 31 spawning events recorded included the release of viable eggs after the release of sperm.

Spawning of tridacnid clams has been induced by the addition of macerated gonads (Wada, 1954; Rosewater, 1965; Hardy and Hardy, 1969; LaBarbera, 1975; Jameson, 1976), raising the temperature of the ambient water (Jameson, 1976; W. Hamner, pers. com.), and by hydrogen peroxide (Beckvar, 1981). Of these three methods, macerated gonads seems to be the most reliable, usually eliciting spawning within 5 min. Peroxide and warm water produce signs of stress in the clams, such as the production of mucus and closing of the shell. Other bivalves have been induced to spawn with chemical and thermal stimulation (see Loosanoff and Davis, 1963). In some cases these spawnings have been normal. In others few eggs are released, subsequent development is often abnormal, and survival of the larvae is poor.

In our experiments, only *T. squamosa* released viable eggs. The two spawnings were spontaneous. All artificial methods induced only the release of sperm and some immature eggs. These results and those of others (see Jameson, 1976) suggest that regardless of the method of induction, the gonads must be mature before normal release of both eggs and sperm can occur.

In the tridacnids, the factors influencing gonad development and the initiation of normal spawning behavior *in situ* are not known. Seasonality, temperature, phases of the moon (tides), and water motion have all been implicated. Of these, a strong case can be made only for seasonality. It appears from our study, and others (see Yamaguchi, 1977), that tridacnid species have different breeding seasons (see Table II). This implies that in nature, different environmental cues may trigger gonad maturation and spawning. Our results indicate that water temperature is probably not a critical factor for the induction of spawning of tridacnids *in situ* (also see Wada, 1954; LaBarbera, 1975).

The evidence concerning the importance of phases of the moon, time of day, tides, and currents to spawning is conflicting (LaBarbera, 1975; Jameson, 1976, Beckvar, 1981). Although spawning may be seasonal for a given species, the actual time span of spawning may vary from one year to another and may occur for only a brief period (Stephenson, 1934; Jameson, 1976; this study). Until factors influencing gonad development are determined, successful induction of normal spawning of eggs and sperm for experimental work or mariculture will depend on the investigator being in the right place at the right time.

Acquisition of zooxanthellae

Many corals, anemones, and hydroids pass their symbiotic algae directly to their offspring in sexual reproduction (Duerden, 1902; Mangan, 1909; Fraser, 1931; Abe, 1937; Kawaguti, 1940; Atoda, 1947a, b, 1951b, c, 1954; Harrigan, 1972). Yonge (1936) originally proposed maternal inheritance of zooxanthellae by tridacnids. However, Stephenson (1934) and Mansour (1946c) found no zooxanthellae in unreleased eggs in *H. hippopus* and *T. maxima* respectively. Our observations corroborate those of LaBarbera (1975) and Jameson (1976) in finding no zooxanthellae in spawned eggs. Each generation must acquire their symbionts from the environment. This phenomenon occurs in a surprising assortment of invertebrates, including the jellyfishes *Cassiopeia andromeda* (Gohar and Eisawy, 1960; Ludwig, 1969; Rahat and Adar, 1980), *Mastigias papua* (Sugiura, 1963, 1964), the gorgonians *Pseudopterogorgia bipinnata, P. elisabethae, Briareum asbestinum* (Kinzie, 1974), the corals *Astrangia danae* (Szmant-Froelich *et al.*, 1980), *Acropora bruggemanni* (Atoda, 1951a) and *Pocillopora meandrina* (Stimson, pers. com.), the anemones *Anthopleura elegantissima* and *A. xanthogrammica* (Siebert, 1974) and *A. tagetes* (G. Muller Parker, pers. com.), and the heart shell *Corculum cardissa* (Kawaguti, 1950).

Uptake and persistance of zooxanthellae in gastrodermal cells of coelenterates involves possible discrimination of strains on surface contact, during phagocytosis, and/or intracellular recognition after phagocytosis (Trench, 1981; Trench *et al.*, 1981). Our results with *T. squamosa* indicate that initial uptake of zooxanthellae is through the mouth and into the stomach, and is nondiscriminatory. Many zooxanthellae are subsequently seen in the viscera. After metamorphosis, zooxanthellae apparently pass from the alimentary system by an unknown mechanism into the developing siphonal tissues, where it often difficult to distinguish by electron micrographs whether they are intra- or extracellular. We interpret this stage as that which indicates the establishment of an association. Clams were able to form an association in this manner with any strain of *S. microadriaticum* introduced after the development of the larval esophagus, stomach, and intestine, These findings imply that in nature, individuals of the same species might not necessarily all contain the same strain of zooxanthellae, unless there is subsequent sorting and elimination of all but one strain, as is seen in reinfection studies on the flatworms *Convoluta roscoffensis* and *Amphiscolops langerhansi* (Provosoli *et al.*, 1968; Taylor, 1971b, 1980).

Tridacnids live in an environment where a number of amphidinioid and gymnodinioid zooxanthellae are potentially available. All reef-building corals and many gorgonians, sea anemones, and jellyfishes harbor *S. microadriaticum* in their tissues (Freudenthal, 1962; Taylor, 1969, 1973, 1974). Under certain conditions zooxanthellae are released from these hosts, often in a healthy vegetative or motile state (Mansour, 1946c; Goreau, 1964; Trench, 1974; Steele, 1975; Ricard and Salvat, 1977; Jaap, 1979; Trench *et al.*, 1981). These algae may be taken into the stomachs of larval, juvenile, or adult tridacnids (Fankboner and Reid, 1981).

When zooxanthellae are outside their hosts, either in seawater or culture media, they alternate between a coccoid non-motile form and a dumbell-shaped motile form (McLaughlin and Zahl, 1957, 1959, 1966; Freudenthal, 1962; Taylor, 1973; Loeblich and Sherley, 1979; Deane *et al.*, 1979). Previous studies on cultured zooxanthellae have shown that motile zooxanthellae are found only in the light phase of a normal light:dark cycle (Fitt *et al.*, 1981). Ricard and Salvat (1977) made similar observations on motile zooxanthellae in fecal pellets from freshly

collected *T. maxima.* Motile zooxanthellae are thought to be important in the infection of potential aposymbiotic hosts (Kinzie, 1974; Steele, 1977; Trench, 1979, 1980). We have shown here that veligers are more likely to take up motile zooxanthellae than non-motile zooxanthellae. Veligers, being planktonic filter feeders, are more likely to encounter swimming rather than sessile algae. The ability of the veligers to take in motile zooxanthellae may be responsible for our differential survival results (Fig. 5, Table III). By this reasoning, the more motile zooxanthellae available, the more will be taken into the gut, with the possible result of more nutrition, leading to higher survival rates. *If* this is the case, intrinsic motility patterns of different strains of zooxanthellae (Fitt *et al.*, 1981) may be very important to the acquisition, nutrition, and survival of juvenile clams in nature. Strains of zooxanthellae which are motile throughout the day are more likely to be taken up (in greater quantities) than strains motile for only half of the day. Unfortunately, the natural diets of larval, juvenile, and adult tridacnids are not known, making it difficult to determine the relative importance of zooxanthellae to their nutrition. Jameson (1976) found that veligers of *H. hippopus, T. maxima*, and *T. crocea* all "fed" primarily on 5 μm flagellated cells when maintained in unfiltered seawater. These cells were not further identified.

Biological specificity in symbiotic associations has been addressed by Weiss (1953), Dubos and Kessler (1963), Schoenberg and Trench (1980c), and Trench *et al.*, (1981). One may consider two levels of specificity in the association between *S. microadriaticum* and the Tridacnidae. The first level concerns the species of algae that live endosymbiotically in tridacnids. Although many species of phytoplankton inhabit coral reef waters, including at least one other symbiotic dinoflagellate (*Amphidinium* sp.), only the dinoflagellate *S. microadriaticum* is found in the tissues of tridacnids. In addition, no adult clams are found without this species of alga. These facts alone imply the existence of a selection process that enables *S. microadriaticum* to establish and maintain a stable relation with its host. Our data suggest possible mechanisms by which selection may take place.

A second level of specificity involves strains of *S. microadriaticum* in tridacnid clams. It is not clear whether there is a selective advantage for clams having a particular strain of *S. microadriaticum*, as suggested in marine coelenterate symbiosis (Schoenberg and Trench, 1980c; Kinzie and Chee, 1979). We found no significant difference in sizes of veligers with different strains of zooxanthellae. However, there was large variability in size and times of development of these veligers. Juveniles infected with different isolates of zooxanthellae demonstrated different growth rates. Kinzie and Chee (1979) found a similar phenomenon with different isolates of zooxanthellae injected into the anemone *Aiptasia pulchella.* Although not apparently important in growth and development of veligers, specific strains of zooxanthellae appear to be important to growth of juveniles and may also influence adult growth and reproductive success. Such is the case with *C. roscoffensis* and *A. langerhansi,* which may harbor many different species of algae, but only develop gonads and spawn with their naturally occurring symbiont (Provosoli *et al.,* 1968; Taylor, 1971b). The growth, survival, and reproductive success of adult tridacnids with different strains of zooxanthellae is not known.

These findings may have practical application. Tridacnid clams were once widespread thoughout the Indo-Pacific, but in the face of intense overfishing in many areas, these clams, particularly the larger members of the group, are facing extinction (Pearson, 1977). Several groups have initiated studies aimed at mariculture of "giant clams", mostly from the point of view of rearing them as a potential food source (Beckvar, 1981; Yamaguchi, 1977; MacIean, 1975). Knowledge of spawning

and development of tridacnids, with particular reference to their association with zooxanthellae, may be important in future attempts at the mariculture of these animals.

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